	Type	L #	Hits	Search Text	DBs	Time Stamp
1	BRS	L1	3	((wheat adj germ adj agglutinin) or (con adj a) or PSA or LCA) same ((neuro$ or neural) near promoter)	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	2005/12/29 10:37

	Type	Hits	Search Text
1	BRS	77	yoshihara and neural
2	BRS	0	yoshihara and neural and trasn-synaptic
3	BRS	4	yoshihara and neural and trans-synaptic
4	BRS	0	(neur? adj promoter)
5	BRS	10	(neural adj promoter)
6	BRS	8538	(neuron and promoter)
7	BRS	5658	(S6 and transgenic)
8	BRS	5457	S7 and mouse
9	BRS	5441	S8 and human
10	BRS	2805	S8 and drosophila
11	BRS	6	(transgenic same gfp same mouse).clm.

	DBs	Time Stamp	Comments	Error Definition
1	US-PGPUB; USPAT; EPO; JPO; DERWENT	2005/12/28 17:00		
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3	US-PGPUB; USPAT; EPO; JPO; DERWENT	2004/08/18 15:58		
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5	US-PGPUB; USPAT; EPO; JPO; DERWENT	2005/12/29 10:33		
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9	US-PGPUB; USPAT; EPO; JPO; DERWENT	2005/04/06 08:00		
10	US-PGPUB; USPAT; EPO; JPO; DERWENT	2005/04/06 08:00		
11	US-PGPUB; USPAT; EPO; JPO; DERWENT	2005/12/28 17:01		

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     CEABA-VTB, CIN, CONFSCI, CROPB, CROPU, DDFB, DDFU, DGENE, DISSABS, DRUGB,
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              2    FILE LIFESCI
              2    FILE MEDLINE
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              2    FILE SCISEARCH
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              1    FILE USPAT2
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L3            4 DUP REM L2 (7 DUPLICATES REMOVED)
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Welcome

Welcome to the Office of Research and Graduate Education and the Pennsylvania Agricultural Experiment Station. Our office serves over 400 faculty and research personnel and, in 2004, facilitated over $89 million in research expenditures. Included in this total are more than 250 Agricultural Experiment Station Projects that our faculty conduct as part of their mission. The College enrolls nearly 500 students from all corners of the world to conduct graduate study and research at Penn State. Our graduate programs are offered in 15 major areas within the College. In addition, students may choose to enroll in 10 interdisciplinary programs that are supported by faculty advisers in the College. We are committed to training the next generation of colleagues in academic, industry, and government positions.

In the coming year, Penn State celebrates 150 years of teaching, research, and service. Agriculture was at the heart of the original Farmer's High School in 1855, and it remains a central feature of Pennsylvania's Land Grant University. Many of our tools have changed, and we train students to perform in areas of science and to address problems that did not exist even a decade ago. Agricultural research addresses critical issues of agro-security, business profitability, environmental impact, youth development, food safety, rural sustainability, resource development and utilization, and natural resource conservation. Our mandate still stands--we have the responsibility to provide a bridge between sound scientific research and the needs of agricultural producers, consumers of our food and fiber products, and rural communities.

Dr. Bruce A. McPheron
Associate Dean of Research and Graduate Education
Director of the Pennsylvania Agricultural Experiment Station

Contact Us

217 Ag Admin. Bldg.
University Park, PA
16802 USA

Phone: 814-865-5410
Fax 814-863-7905
Email:
agresearch@psu.edu

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PENNSTATE

RESEARCH PROJECT OUTLINE

Research and Graduate Education ♦ College of Agricultural Sciences
The Pennsylvania State University ♦ University Park, Pennsylvania

Title: Transgenic Mouse Models for Studying Mammalian Development and Disease

Probable Duration: 57 months (April 1, 2005- December 31, 2009)

Personnel:

Project Leader:
Cooduvalli S Shashikant, 85%
Dairy and Animal Sciences

Justification, Relevance, and Expected Outcome or Impacts:

Transcriptional regulation is one of the key mechanisms that control mammalian development. Sequential activation of target genes by transcription factors progressively determines how a one-cell embryo develops into the whole organism. Mechanistically, it is critical to understand how regulatory interactions shape embryonic body axes. This requires identification of critical regulatory sequences of developmental control genes and factors, which interact with these regulatory sequences. In this study, we propose to analyze regulatory interactions of homeobox genes. Homeobox genes are present in most metazoans studied so far. Many of these genes show remarkable conservation of sequence, structural organization and function among wide number of species studied so far. Yet, remarkable morphological diversity among metazoans suggests that specific modifications in the homeobox gene system have occurred in different taxa. Hence, a comparative study of homeobox genes and regulation will provide critical information on morphological diversity.

For studying mammalian development, it is desirable that such investigations are carried out in the context of whole organism. Such an approach is feasible in the mouse. The mouse genome can be easily manipulated by transgenic methods. The effect of such manipulations can be assayed effectively in developing embryos. Transgenic based approaches will be extensively used in the characterization of regulatory interactions of homeobox genes. Many of these homeobox genes are organized in clusters. Clustered homeobox genes, Hox genes are regulated in a complex manner. The individual Hox gene expression is likely to be controlled by regulatory elements present throughout the cluster. Studying interactions among cis-regulatory elements scattered in the cluster is a difficult task. This problem can be approached using recombinogenic targeted methods described in this proposal. Using these approaches, large-insert constructs carrying Hoxc cluster will be modified to study contribution of specific cis-acting elements in the context of the cluster.

Finally, transgenic mice provide excellent models for human pathologies. Recombinogenic targeting methods developed in this group are very useful in manipulating large-insert constructs to generate transgenic mice that express candidate disease genes more close to endogenous gene expression. This approach can be used as a way of illustration in generating mouse models that mimic human pathologies., such as polycystic kidney disease and neurodegenerative disorders. Transgenesis also leads to insertional mutagenesis. Transgene often disrupts genes resulting in mutant phenotypes. Since these disruptions are at random locus, the chances of finding new genes with an evident phenotype are relatively high. Thus, transgenic mice can be useful in identifying genes that are important for normal development and disease.

The project outlined here is expected to make significant contributions towards understanding fundamental processes involved in animal development and provide useful models for studying animal diseases.

Previous Work (Background) and Present Outlook:

The isolation of developmental control genes has dramatically improved our understanding of the genetic interactions and molecular mechanisms that control the transformation from fertilized egg to patterned embryo. The study of homeobox containing genes first identified in *Drosophila* and subsequently in other experimental organisms has established transcriptional regulation as one of the key mechanisms by which early developmental decisions are made (for reviews see: Krumlauf 1994). More than 100 homeobox genes have been identified among metazoans. Thirty-nine of these form a distinct class of clustered genes, termed Hox genes, which share sequence homology with the *Drosophila* Hom-C cluster. The clustered organization of Hox genes arose as a consequence of gene and genome duplication during evolution. Invertebrates have a single cluster of Hox genes with a variable gene number (*Drosophila* has 8 genes, amphioxus has 14). In contrast, vertebrates have several Hox clusters as the result of whole-cluster duplications. Tetrapods, including mouse and human, have four Hox clusters on four separate chromosomes, which were generated by at least two large-scale duplication events. Another duplication event in the lineage that leads to teleosts has led to the presence of more than four Hox clusters in this group, with pufferfish and zebrafish having seven clusters (Amores et al., 1998; Amores et al., 2004). Consistent with the evolutionary relationship arising from duplication events, Hox genes show a remarkable conservation in their organization, expression and function. Orthologous genes have highly conserved functional domains (homeodomain) and often share very similar regulatory modules.

Hox genes share several striking features, which can be best described in terms of collinearity (Kmita and Duboule, 2003). Genes located at the 3' end of the cluster are activated earlier during embryogenesis, are more sensitive to retinoic acid (RA), and are expressed in more anterior regions of the embryo. Genes located at the 5' end of the cluster are activated later during embryogenesis, are less sensitive to RA and function in more posterior regions of the embryo. Certain regulatory constraints perhaps imposed physical constraints, resulting in the conservation of the clustered organization of Hox genes. Partial overlap in domains of Hox gene expression along the embryonic anteroposterior axis generates a Hox code, which imparts positional information. Experimental manipulations, which alter the expression of Hox genes, have led to alterations in the identity of axial structures in several organisms studied so far (Krumlauf, 1994). The clustered organization of Hox genes may be critical to their coordinated expression along the primary or secondary body axes. Although the biological relevance of the coordinated gene expression is clear, the mechanisms by which this transcriptional progression is achieved among genes organized in cis are not well understood. A significant insight into this problem has been gained by experimental manipulations carried out in Denis Dubloule's laboratory, aimed at changing the position of a gene within the cluster (Kmita and Duboule, 2003; Spitz et al., 2003). These experiments suggest that spatial and temporal expression of a gene is partly dependent on its position within the cluster. Similar regulatory mechanisms of other Hox complexes have yet to be identified.

Although the coordinated expression of clustered genes may be orchestrated by global regulatory mechanisms, major aspects of individual Hox gene expression are controlled by cis-acting elements flanking each gene. Isolated Hox genomic loci can confer an expression pattern to a reporter gene in transgenic mice that recapitulates the endogenous gene expression pattern (reviewed in Krumlauf, 1994; Lufkin, 1996). Cis-acting elements located in the vicinity of the transcriptional unit also function outside of the cluster. This is evident by the expression patterns of Hox reporter transgenes that integrate randomly in the genome. A major shortcoming of studies on regulation of Hox genes and complexes is that their cis-acting sequences are poorly characterized, compared with those of viral genes. Potential site interactions for a few known transactivators have been determined (reviewed in Krumlauf, 1994; Lufkin, 1996). However, there remains few detailed and systematic analysis of cis-acting elements that regulate Hox gene expression. Our studies on the early enhancer of *Hoxc8* have identified about nine separate *cis*-acting elements within a 200 bp fragment, which interact in a combinatorial fashion to generate the early phase of *Hoxc8* expression (Shashikant et al., 1995;

Shashikant and Ruddle, 1996; Wang et al., 2004). These studies not only suggest potential trans-activators involved in the regulation of *Hoxc8*, but also provide a basis for understanding how *cis*-regulatory elements have contributed to morphologic diversity (Belting et al., 1998a; Shashikant et al., 1998b; Anand et al., 2003; reviewed in Holland, 1999; Carroll, 2000; Tautz, 2000). Furthermore, a detailed characterization of cis-acting elements is essential to understand transcriptional regulatory mechanisms underlying cis-interactions that contribute to the organization and function of Hox complexes.

Mouse models have become increasingly valuable for studying neurodegeneration. Many mutations have been identified as a result of insertional mutagenesis via the introduction of exogenous DNA in the germ line of mice either by a gene trap or a transgenic method. In addition to causing the mutation, the transgene serves as a tag for molecular cloning of the affected locus. A number of mutations such as *rostral cerebellar malformation (rcm), hotfoot (ho), twitcher (twi), and pcd* have been produced by transgene insertion (Meisler, 1992). Mutated genes have been cloned in many instances (Singh et al., 1991; Keller et al., 1994; Kohrman et al., 1995; Perry III et al., 1995; Beier et al., 1996). Transgenic mouse models for human disorders including Down syndrome, MJD/SCA3, HD, SCA1 and AD have provided information about the cellular and molecular mechanisms responsible for the neuropathology of these diseases (Orr and Zoghbi, 1999; Borchelt et al., 1999, Baxter et al., 2000). We have undertaken an initial characterization of a novel mouse model *Jitters*. *Jitters* arose as a result of insertion of a *Hoxc8-lacZ* transgene in the mouse genome. *Jitters* exhibits a wide range of abnormalities in the central nervous system (CNS) including loss of cholinergic cells in the basal ganglia, loss of proliferative cells in the neurogenic regions of the brain and loss of granule cells in specific cerebellar lobes. We propose to determine the genetic and molecular pathway underlying this mutant phenotype. We will focus on the involvement of neurotrophin mediated pathways of cell loss in different regions of the CNS. We will also identify the genetic basis for the *Jitters* phenotype. Molecular analysis suggests that the *Hoxc8-lacZ* transgene is linked to a genomic locus that contains sequences homologous to *glyceraldehhyde-3-phosphate dehydrogenase (GAPDH)*. GAPDH has been traditionally considered to be a 'house-keeping' gene that functions in glycolytic pathways of energy metabolism. However, recent studies implicate GAPDH in neurodegeneration, leading to development of strategies for pharmacological interventions.

Objectives:

A long-term goal of my laboratory is to describe regulatory relationships among various genes that control animal growth and development. Since homeobox containing genes play critical role in controlling developmental processes, a major aim of this research project is to understand the regulation of homeobox genes in the context of embryonic development and organ specification. A combination of transgenic, embryonic, molecular and biochemical techniques will be used to characterize transcriptional regulatory network that controls developmental processes.

The strength of my laboratory lies in the use of transgenic based technologies in addressing problems related to development. Specialized methods developed in the group such as recombinogenic targeting techniques will be used to generate sophisticated transgenic mouse models. Efforts will be made to characterize insertional mutations that arise in transgenic colonies. These investigations can be described under four approaches described below.

1. *To identify critical cis-acting elements of Hoxc8 early enhancer that determine axial level of Hox gene expression.*
2. *To identify critical trans-acting factors that interact with enhancer elements in determinin gaxial level of Hox gene expression*
3. *To determine the consequence of the variation in Hoxc8 early enhancer elements on axial patterning.*
4. *To develop transgenic mouse models for studying selected human pathologies such as polycystic kidney disease and neurodegenerative disorders.*

Procedures:

1. Cis-regulatory analysis

The purpose of this study is to refine our analysis of *Hoxc8* early enhancer to identify new elements within the 200 bp *Hoxc8* early enhancer. We will use phylogenetic comparison as a basis for examining new sequences. Mutations will be introduced in critical sequences and their effect will be measured by reporter gene analysis in transgenic mouse embryos. To date, we have identified nine elements (A-I) by mutational analysis of the *Hoxc8* early enhancer using this approach. Comparison of *Hoxc8* early enhancer sequences isolated from different vertebrates including mouse, chick, coelacanth, puffer fish and zebrafish identify additional blocks of highly conserved regions. Furthermore, the diverged sequence between mammal and fish will be searched for additional cis-acting elements. Sequences critical for enhancer activity within these conserved regions will be identified by a combination of DNA mobility shift assays using protein extracts from mouse embryos and mutational studies in reporter gene assays in transgenic mouse embryos. The nature of combinatorial interactions at these newly defined sites will be determined by constructing enhancers carrying different combinations of multiple mutations and further tested by reporter gene analysis. This experimental system will facilitate the identification of motifs that regulate *Hoxc8* expression in different sub-regions of the developing mouse embryo. The regulatory motifs, in turn, will help us to identify transcription factors controlling *Hoxc8* expression during early embryonic development.

The specific question to be addressed in this aim relates to the mechanisms that control different axial levels of Hox gene expression. What determines the anterior boundary of expression? Since Hox genes are expressed to different axial levels along the embryonic axis, it is critical to understand how differential boundaries are set for each Hox gene. From an evolutionary perspective, this question may provide clues to interspecies difference in axial levels of Hox gene expression, which is in turn related to changes in axial morphologies. *Our hypothesis is that different combinations of elements contribute to anterior extent of expression.* A comparison of fish and mouse elements will provide insights on transcriptional machinery present in the mouse that can discriminate between two enhancers activities and how they relate to different axial levels of expression. Transgenic mouse embryo can be used as an 'assay system' to determine changes in cis-acting elements that alter spatial patterns of gene expression. In our experience, 'transient transgenics' provides a rapid method of analysis or cis-regulatory sequences and provide direct evidence for the critical elements that govern Hox gene expression during embryonic development. The PI has extensive experience in transgenic methods and the analysis of Hox gene regulation.

Transgenic mouse technology is a powerful and efficient tool for analysis of cis-regulatory elements of developmental control genes. Most of the analysis of reporter gene expression will be carried out in days 8.5-9.5 founder generation embryos. This type of analysis, often referred to as a 'transient assay', allows a number of reporter gene constructs to be examined in a relatively short time. At these stages, reporter gene activity can be readily visualized by wholemount staining procedures. This type of analysis often referred to as a 'transient assay' allows a number of transgene constructs to be examined in a relatively short time. Transient assays have been successfully employed in the analysis of the Hoxc8 early enhancer. In a typical experiment, 2-5 embryos expressing reporter gene activities are obtained per injection session, which enable a rapid deletion/mutation analysis of the given regulatory region. The transient analysis provides a rapid method for cis-regulatory analysis of Hox genes. This method is rapid, efficient and relatively inexpensive for performing the deletion/mutation analysis described in this study.

Critical nucleotide sequences within *Hoxc8* will be identified by DNA-protein binding studies using protein extracts obtained from day 9.5 day mouse embryos. Sequences important for protein binding will be mutated and further tested in transgenic system for their contribution to the enhancer activity. The regions that will be tested for protein binding includes PFP1-3; Fish-1 and 3'-diverged fish enhancer sequences. In our previous studies, we have used this approach to identify elements A, B, D and F (Shashikant et al., 1995; Wang et al., 2004). Total protein extracts prepared from day 9.5 embryos provide a good source for assaying sequence-specific DNA binding proteins by standard mobility shift assays. In addition to DNA-protein binding studies, transcription factor databases will be searched for potential factors capable of interacting with the PFP1-3 sequences. A recent search through such databases did not yield any significant information on

potential binding proteins to PFP-1, PFP-3 or diverged *Fugu* and zebrafish sequences. However, PFP-2 was shown to contain a core sequence (AGACGTCT) important for interactions with Smad4 proteins (Zawel et al., 1998). Sequences necessary for Smad4 binding will be tested for their ability to bind to proteins by competition assays. DNA-protein binding studies using mouse embryo extract provides necessary guidance for the identification of critical nucleotides that contribute to enhancer activity.

The activity of the *Hoxc8* early enhancer will be assayed in the context of a heterologous promoter or heat shock protein 68, hsp68 linked to bacterial β-galactosidase gene with an SV40 polyA signal (Kothary et al., 1989). The *hsp68* promoter provides sequences required for basal transcription. On its own, this promoter has very little activity in transgenic mouse embryos but it responds to the enhancers cloned in its vicinity. The *Hoxc8* early enhancer cloned upstream of the hsp68 promoter directs the activity of the β-galactosidase gene in a spatially restricted pattern in day 9.5 embryos as shown in the previous section. The reporter cassette will be digested with appropriate restriction enzymes to release the transgene insert free from vector fragments. The insert will be purified on a sucrose density gradient and dialyzed against injection buffer (Shashikant et al., 1995). Mutations in the 200 bp early enhancer sequence will be introduced by an overlapping PCR based strategy using synthetic oligonucleotide primers containing appropriate changes in the nucleotide sequence (Shashikant et al., 1995). In general transverse substitutions will be made in the potential cis-regulatory sequences (for instance A to C; G to T, and vice versa). The mutated fragment will be sequenced to confirm the specific changes made in the sequence, as we must ensure that no unintended changes are made in the modified early enhancer sequences.

Critical nucleotide sequences that affect protein binding in the area of *PFP-1-3* will be tested for their effect on *Hoxc8* early enhancer activity. The reporter gene expression will be assayed in day 9.5 transgenic mouse embryos. The expression in posterior neural tube and mesoderm will be determined. Mutations introduced at a single site often fail to affect enhancer activity on their own, as in the case of mutating site B, for example (Shashikant and Ruddle, 1996). The significance of some of the sites becomes evident when other sites are simultaneously mutated. In the case that single mutations at sites PFP-1, 2 or 3 do not affect *Hoxc8* early enhancer activity, mutations will be examined in combination with other mutated elements. These studies are generally carried out in a sequential manner, with each result providing insights on the design of the next mutation. It is important to generate a series of double, and even triple site mutations involving PFP1-3 to completely understand the nature of the combinatorial interactions that determine *Hoxc8* early enhancer activity. In this series of experiments, we plan to examine three reporter constructs for single mutations and about ten reporter constructs for mutations at different sites.

The experiments outlined above will provide further insights into critical elements contained within the *Hoxc8* early enhancer. Although these studies are guided by phylogenetic comparisons, the existing repertoire of mutations in the *Hoxc8* early enhancer allows us to make judicious changes in the new sequences, which will affect reporter gene expression in the neural tube, somites and lateral plate mesoderm. These elements may affect both anterior extent and tissue specific expression patterns of the reporter gene. The nucleotide sequence of PFP-2 contains a potential binding site for Smad4 proteins. In the literature, there is a growing body of evidence suggesting interactions between Smad and Hoxc8 proteins in the regulation of downstream targets involved in bone morphogenesis (Li and Cao, 2003). If Smad4 is implicated in Hoxc8 regulation, it provides an interesting insight into a potential regulatory loop controlling the transcriptional regulation of osteoblast differentiation. PFP-1 and 3 may represent binding sites for novel transcription factors.

2. Trans-acting factors interacting with Hoc8 early enhancers

The main purpose of this study is to establish experimental systems for studying the transcriptional regulation of *Hoxc8*. The *Hoxc8* early enhancer is one of the most characterized mammalian Hox regulatory regions to date. Because of the *in vivo* methods used for the identification of cis-acting elements, a wealth of information exists on the effects of mutating these elements on tissue and region-specific expression patterns. Critical elements for the enhancer activities have been mapped at the resolution of 2-3 nucleotides. A large number of enhancer constructs, each carrying different combinations of mutations in their elements, already

exists in the PI's laboratory. Potential transcription factors capable of interacting at these sites have been suggested in the literature. A fair amount of evidence suggests that Cdx proteins are upstream regulators of Hox genes, and a number of reagents required to link Cdx proteins to the regulation of *Hoxc8* have been generated in the laboratory. However, lack of *in vitro* systems to study transcriptional regulation of Hox genes as relevant to early embryogenesis has greatly hampered progress in the field. To date, we have screened a number of cell culture systems and found that none support *Hoxc8* early enhancer activity and are thus unsuitable for transcriptional studies (Shashikant, unpublished observations). This shortcoming can be addressed by a three-pronged approach. First, we will establish immortalized cell lines derived from transgenic mouse embryos carrying a *Hoxc8-lacZ* transgene and screen for those cell lines that retain *Hoxc8* early enhancer activity. Second, we will use *Cdx* genes as 'proof-of-principle' to study regulation of *Hoxc8* regulation *in vitro*. Activator and repressor constructs of Cdx genes will be generated and tested for their effect on *Hoxc8* early enhancer activity. siRNA molecules will be developed against Cdx genes test their ability to alter *Hoxc8* enhancer activity by abrogating Cdx expression. Third, we will construct cDNA libraries from immortalized cell lines and/or cell populations derived from the posterior regions of day 8.5/9.5 mouse embryos using PCR based cDNA synthesis and cloning methods. These libraries will be screened by one-hybrid or two-hybrid methods for proteins capable of interacting with *Hoxc8* early enhancer elements. With these resources and methodologies, we will be able to establish an *in vitro* system for the study of *Hoxc8* regulation. These studies will provide a bridge between *in vitro* and *in vivo* studies on Hox gene regulation. Results obtained from these experiments can readily be confirmed by direct manipulation of mouse embryos by a transgenic approach.

We will explore the possibility of establishing proliferative cells from the caudal regions of day 9.5 embryos. Differentiated cells when explanted to the *in vitro* environment frequently extinguish their specialized epigenetic patterns of gene expression. However, it has been shown in an increasing number of instances that dedifferentiation can be circumvented to varying degrees by neoplastic transformation using carcinogens, oncogene expression, oncogenic viruses or the *Telomerase* gene. We will attempt to immortalize specialized cells derived from the posterior regions of the embryo. Key to our approach will be the use of reporter gene expression, which will serve as a 'tag' to recognize and select for cells that support *Hoxc8* early enhancer activity. Embryonic cells expressing the reporter gene specific for a particular cell type will be explanted *in vitro* and transformed using an *hTRT* (*human telomerase reverse transcriptase*) construct. Exogenous introduction of the catalytic subunit of *hTRT* has been shown to be successful in the *in vitro* immortaization of numerous cell types, including primary human mammary epithelial cells (Kim et al, 2002) and can extend the proliferative capacity of other cell types, such as primary human myoblasts (Donna et al, 2003). In our experimental system we will use reporter gene expression as a measure of retention of differentiated state. This trait can be selected by inspection using replica colony assay and/or cell sorting insuring a high level of quality control. The isolation of differentiated cell populations can be useful in a number of different ways, including cotransformation experiments using a similar enhancer coupled to a different reporter such as luciferase, and in combination with other primary embryonic cells of specific type to test for intercellular signaling.

Immortalized cell lines established by above methods will be used to test regulatory relationship between Cdx and *Hoxc8*. As outlined in the preliminary studies, data from knockout studies, DNA-protein binding, and overlapping expression patterns argue for a role for Cdx proteins in regulating different aspects of early *Hoxc8* expression. Consistent with these observations, *Hoxc8* early enhancers contain at least two caudal binding sites (A and D). However, a direct regulatory relationship between Cdx and *Hoxc8* has not yet been established. We have generated activator and repressor forms of all three Cdx proteins. These constructs will be transfected into *Hoxc8-lacZ* expressing cell lines and their effect on the enhancer activity will be quantitated by using a chemi-luminoscent method for detection of b-galactosidase activity using Galacto-Light Plus kit (Tropix Inc; Jain and Magrath, 1991). These studies will validate the usefulness of cell lines for studying *Hoxc8* regulation.

We will establish RNA interference-based methods to screen candidate transcription factors regulating Hoxc8 early enhancer activity RNA interference (RNAi) is a gene silencing method that results in the destruction of a selected target messenger RNA (mRNA) with high precision and efficiency (reviewed in

McManus and Sharp, 2002). RNAi leads to the cleavage of target mRNA following binding of a 21-nucleotide (nt) short interfering RNA (siRNA) to the targeted mRNA (Elbashir et al., 2001). siRNA can be chemically synthesized and delivered to cells. However, this method requires high cost and is impractical. Instead, transgenes that will express siRNA upon transcription can be incorporated into the host genome, resulting in the stable expression of siRNA, leading to prolonged gene silencing. Upon integration into the host genome, the transgenes produce an intermediary double stranded RNA structure called stem-loop siRNA. Within the cells, stem-loop siRNA is cleaved to form siRNA, which later binds to and inactivates the target mRNA (Fire, 1998; Hammond et al., 2001). RNAi has been used to knock down (silence) specific target genes in a wide range of organisms both in vitro and in vivo. Very low concentrations of siRNA introduced into neuronal cell cultures have been shown to suppress endogenous and heterologous genes (Krichevsky and Kosik, 2002). In addition, siRNAs have been found to silence specific target genes in mammalian cell lines such as human embryonic kidney cells and HeLa cells (Elbashir et al, 2001). A luciferase gene specific siRNA has also been demonstrated to suppress the expression and activity of a luciferase transgene in adult mice (McCaffrey et al., 2002). From the foregoing, it is evident that RNAi is evolving as a promising tool for silencing gene function at the post-transcriptional level both in vitro and in vivo.

One of the challenges in knocking down target mRNA is stable incorporation of the siRNA transgene in the host genome and sustained expression of the siRNA in high concentrations to destroy the target mRNA. As lentiviral vectors have considerable advantages in conferring stable expression of siRNA, we will develop a lentiviral vector to integrate siRNA for knocking down the lacZ gene. The lentiviral vector is a hybrid viral vector that is replication incompetent and non-infectious to the host animal (Naldini et al., 1996; Dull et al., 1998; Trono, 2000; Tsui et al., 2002). The lentiviral vector has a unique advantage of conferring stable incorporation of cloned genes, even in non-dividing cells such as neurons. It is derived from the human immunodeficiency virus (HIV-1) genome wherein only three of the nine genes present in the HIV-1 are retained while six genes are deleted. The remaining three genes are gag, which codes for the virion main structural protein, pol, which codes for retroviral specific enzymes, and rev, which codes for an efficient post-transcriptional regulator necessary for efficient gag and pol expression. The lentiviral vector is packaged in an envelope from a heterologous virus glycoprotein (vesicular stomatitis virus). Due to such an extensively deleted packaging system, the parental virus (HIV-1) cannot be reconstituted from the lentiviral vector used for gene delivery, as 60% of the HIV-1 genome has been completely eliminated. Therefore, the lentiviral vector offers a high level of biosafety to both the laboratory animals and to the laboratory personnel who handle them, and offers great potential for the stable expression of foreign genes. We will establish RNAi based methods to inactivate Cdx gene expression and measure its effect on Hoxc8 early enhancer by standard methods of transfections/infections. Once 'proof-of-principle' is established, we will develop the system to examine other trans-acting factors in regulating Hoxc8 expression.

3. Enhancer modification and axial patterning

Reporter gene analyses, as informative as they are do not provide information on how evolutionary divergence of the *Hoxc8* early enhancer affect endogenous *Hoxc8* expression and hence axial patterning. The developmental attributes of the early enhancer modifications can be understood if these changes are introduced in the context of the endogenous *Hoxc8* gene. How does the replacement of the mouse enhancer by the fish enhancer affect thoracic specification? What is the evolutionary significance of changes in the skeletal identity? How does this affect the early phase of *Hoxc8* expression? Does this substitution induce heterochrony and anteriorization of *Hoxc8* expression? How does this affect the maintenance phase of *Hoxc8* expression? Are there any changes in the expression of the neighboring *Hoxc9* gene? These questions can be answered by a knock-in strategy in the mouse using Cre/loxPrecombination. Replacing the mouse *Hoxc8* early enhancer sequence with the corresponding sequences from the *Fugu Hoxc8* early enhancer by gene targeting would result in an altered mouse with potentially changed *Hoxc8* expression and axial morphology. If the first two specific aims identify elements/factors in the mouse that discriminates two enhancer activities, this aim will provide a direct correlation to changes in the *Hoxc8* expression and aspects of axial specification. Together, we will gain considerable insights on how restructuring of the *Hoxc8* early enhancer potentially alters the axial specification in the mouse. Of the three fish *Hoxc8* early enhancer sequences

examined, *Fugu* enhancer anteriorizes mesoderm expression. For this reason, we propose to knock in *Fugu* enhancer sequences hoping to achieve gain-of-function type alteration in the mouse.

Briefly, as described below, knock in involves (i) generation of a targeting construct containing a modified enhancer region flanked by loxP sites with a neomycin resistance (neoR) gene for positive selection and a Diphtheria toxin (DTA) gene for negative selection; (ii) electroporation of mouse embryonic stem(ES) cells with the chimeric *Fugu*/mouse *Hoxc8* targeting construct and isolation of ES cell clones containing the targeting construct integrated at homologous sites; (iii) generation of chimeric mice containing mutated ES cells by blastocyst transfer; and (iv) identification of mouse lines which transmit the altered enhancer region and interbreeding these to generate mice that are homozygous/heterozygous for the targeted modifications. Cre/loxP technology for creating tissue-specific gene knockouts is now routinely practiced in a number of laboratories and can be considered a standard gene targeting procedure (reviewed in Lobe and Nagy, 1998). Reagents required for the generation off loxed (flanked by loxP sites) Hoxc8 enhancers and Cre recombinase are available in the laboratory. We have mouse genomic fragments containing the entire *Hoxc8* cluster and extensive sequence in formation of the intergenic region between *Hoxc9-Hoxc8* required for the manipulations.

Gene targeting will be achieved by standard methods with the help of PSU transgenic core facility. Mice homozygous for the modified enhancer will be examined for changes in the expression pattern ofHoxc8 and patterning of the vertebral axis. Since the early enhancer is involved in the activation of Hoxc8and establishment of its spatial domain, we will initially examine day 8-9.5 embryos for Hoxc8 expression by whole mount immunohistochemistry. We have produced a panel of monoclonal antibodies against *Hoxc8* and extensively used these antibodies to characterize Hoxc8 expression during mouse embryogenesis (Shashikant et al., 1995; Bradshaw et al., 1996; Belting et al., 1998a, b). These antibodies can be used in immunohistochemical analysis on tissue sections and whole mounts. Early stage embryos (8.0-10.0) can be conveniently examined by whole mount immunohistochemical staining for alterations in anterior boundaries of expression in the neural tube and mesoderm. Both *Hoxc8* RNA and protein distribution will be compared in mutant embryos to determine to what extent transcription and translation of *Hoxc8* is affected by enhancer modifications. Hoxc8 endogenous expression in the wild type mouse is first detected in day 8.0 embryos with 7-8 somites, prior to the turning of the embryos (Belting et al., 1998b). Expression is diffuse in the extreme caudal portion of the embryo with the highest level found at the base of the allantois, an area corresponding to the remainder of the primitive streak. This expression spreads gradually, and in embryos with 10-12 somites, *Hoxc8* exhibits a characteristic out-of-register expression described for mouse Hox genes, with a more anterior expression boundary in the neural tube than in the mesoderm. The anterior boundary of expression in the neural tube at this stage is at the level of the 9th or 10th somite, whereas the expression in the mesoderm is located more posteriorly in the still unsegmented region. At a slightly later stage, in embryos with 16-17somites, expression extends through the entire tailbud. At these stages, expression of the mouse early enhancer-directed lacZ reporter gene strongly resembles endogenous *Hoxc8* expression (Shashikant et al.,1995; Belting et al., 1998b). Transgenic mouse embryos carrying zebrafish enhancer-directed lacZ gene will be stained for β-galactosidase activity with X-gal (Shashikant et al., 1995). The expression pattern of the reporter gene in transgenic embryos will be compared with that of *Hoxc8* in targeted mouse embryos. Based on reporter gene analyses, we predict that replacement of the mouse enhancer with *Fugu* enhancer element results in delayed activation of Hoxc8 with subsequent posteriorization of expression boundary in mesoderm in the mutatedembryos. To date, studies on the *Fugu Hoxc8* early enhancer directed reporter gene have been limited to examination of founder generation embryos at day 9.5. Permanent transgenic lines with this construct will be established to compare temporal sequence of reporter gene expression with that of *Hoxc8* in mutant mouse embryos carrying *Fugu* early enhancer sequences.

Phenotypic changes in the axial patterning in mutant embryos carrying enhancer modifications will be studied first by visible inspection of the early embryos. Patterns of somite condensation, position of hindlimbs and forelimbs, distance between the two limbs and length of tail region will provide clues regarding early malformations resulting from alterations in expression of Hoxc8. Skeletal preparations of the newborn mutant mice will be examined for skeletal abnormalities. We do not expect enhancer modification to

cause embryonic lethality, because homozygous disruption of the Hoxc8 coding region by gene targeting methods yields new born pups, a few of which survive into adulthood (LeMouellic et al., 1992). Newborn pups from mice homozygous for enhancer modifications will be eviscerated and dehydrated with ethanol and acetone. The skeletons will be cleared with KOH and stained with alizarin red by standard procedures. The axial skeleton in the thoracic and lumbar regions will be examined for homeotic transformations transformations and other modifications. In *Hoxc8* knockout mice, the first lumbar vertebra is transformed into a thoracic vertebra with a pair of ribs. Other modifications include the appearance of additional sternal vertebra between the 6th and 7th ribs and fusion of the 8th rib with the sternum. Modifications of the T12 vertebra were also observed with variable penetrance (Le Mouellic et al., 1992). Since *Fugu* enhancer anteriorizes mesoderm expression, we expect to see transformation in more anterior regions of the mutant mice. Since experimental evidence suggests that the axial fate of somites is already established in presomitic mesoderm, *Fugu* early enhancer substitution should affect various aspects of thoracic specification. Since anterioriztion of expression is mostly seen in the mesdoderm, relationship between neural tube and somites could be altered. In surviving mice, LeMouellic et al observed neurological defects manifested in by a clenched fist phenotype (LeMouellic et al., 1992). The motor neurons innervating distal muscles of the forepaw fail to do so in the *Hoxc8* null mutant mice (Tiret et al., 1998). We have shown by a combination of retrograde labeling with DiI and staining with *Hoxc8* antibodies in mouse embryos that *Hoxc8* expressing neurons innervate the brachial plexus (Belting et al., 1998b). We expect that enhancer modification result in confused innervation patterns. These studies, in conjunction with reporter gene analysis, will provide substantial information on how cis-regulatory changes affect axial patterning in the mouse.

4. Recombinogenic targeting and transgenic mouse models for diseases

a. Recombinogenic Targeting

We have developed a technology, the pClasper system, by which large genomic DNA fragments, can be isolated and modified by homologous recombination (Bradshaw et al., 1995; Shashikant et al., 1998). Constructs prepared by our methods are large, on the order of 150-300 kb, and as such, contain substantially all the regulatory elements that control the authentic expression of the gene. Large constructs of this magnitude can be used to generate transgenic mice and effect of different regulatory regions on gene expression can be monitored in the same construct.

This approach will be used to study multiple regulatory interactions occurring in the *Hoxc8* genomic region. A large genomic fragment (30 Kb) containing *Hoxc9-Hoxc6* intergenic region will be captured in pClasper by homologous recombination in yeast. This construct will be modified to insert β-galactosidase reporter gene in frame of the first exon. The reporter gene construct thus generated will be analyzed for its expression in different stages of developing embryos (day 8-13). Several modifications will be made in this reporter construct. For instance, the 200 bp Hoxc8 early enhancer region will be replaced with the homologous enhancers isolated from *Fugu*, zebrafish, chick , coelacanth and other organisms. These replacements will be carried out by recombinogenic targeting methods with no selectable markers incorporated. Thus resulting constructs will carry no extraneous sequences. This will enable us to examine the effect of changes in enhancer sequences on the late phase Hoxc8 expression. This strategy can be used to determine complex interactions occurring between different regulatory modules that control different aspects of Hoxc8 expression during embryonic development.

Similar methods will be used to generate large-insert constructs to create mouse models that are useful in studying human pathologies. A case in study is autosomal dominant polycystic kidney disease (ADPKD) in human. ADPKD is a progressive disease, affecting a high proportion of the population, frequently culminating in complete kidney failure in later life. Currently no successful medical treatment for PKD is available. Our approach centers on the development of authentic models of human ADPKD in transgenic mice. Large genomic fragment containing PKD1 gene will be captured in pClasper by homologous recombination. These inserts will be used to generate transgenic mice expressing human PKD1 gene. Natural mutations in PKD1, occurring in human population will be introduced in the PKD1 genomic construct by recombinogenic targeting. Mutant PKD1 genes will be used to generate transgenic mice. These mice will be characterized for the progression of PKD.

b. Insertion Mutation

Mouse models have become increasingly valuable for studying neurodegeneration. Many mutations have been identified as a result of insertional mutagenesis via the introduction of exogenous DNA in the germ line of mice either by a gene trap or a transgenic method. In addition to causing the mutation, the transgene serves as a tag for molecular cloning of the affected locus. In this study, we propose to develop a novel mouse model for studying neurodegenerative diseases. Recent investigations in medical genetics have identified specific genetic loci affected in a number of neurodegenerative disorders. Mouse models are being used to study etiological factors and pathogenic mechanisms. We describe *Jitters*, a novel mouse model for neurdoegeneration that arose as a result of insertion of a *Hoxc8-lacZ* transgene in the mouse genome. *Jitters* exhibit a wide range of abnormalities in the central nervous system (CNS) including loss of cholinergic cells in the basal ganglia, loss of proliferative cells in the neurogenic regions of the brain and loss of granule cells in specific cerebellar lobes. It is rather uncommon to see such a wide range of abnormalities of CNS in a single mouse and hence studies we propose are important in understanding the role of the disrupted gene in some of the fundamental molecular events that could be common to many neurodegenerative processes. These abnormalities are implicated in Schizophrenia, Alzheimer's disease (AD) and various movement disorders. Experiments will be carried out to determine genetic and molecular pathways underlying this mutant phenotype. In particular the role of neurotrophic factors in the cell loss will be investigated.

The structure of the genomic locus disrupted in *Jitters* will be determined by the molecular analysis of λ phage clones carrying DNA fragments surrounding the transgene insertion. We have already constructed a *Jitters* 'genomic' library from partially-digested and size fractionated genomic DNA. From this library, we have isolated several clones carrying inserts ranging in size from 8-23 kb. At present, we are concentrating on characterization of two λ-phage clones (3 and 5) that contains sequences related to GAPDH in addition to sequences from the *Hoxc8* early enhancer-*LacZ* transgene (confirmed by PCR). We will determine the nucleotide sequence of the fragments cloned in λ-phage clones 3 and 5. Partial sequence obtained for these clones show strong homology to GAPDH coding regions. Sequencing will be carried out from both ends of the inserts to the junction of the transgene sequences. Although λ-phage clones are useful vectors for library construction, it is difficult to isolate high quality DNA in sufficient amounts for sequencing multiple clones. For this reason, insert DNA will be digested with the appropriate restriction enzyme (*Bam* HI) and smaller fragments obtained will be subcloned into a plasmid vector suitable for sequencing (pBluescript). Digestion of λ clones 3 and 5 releases 2-8 kb fragments. Each of these clones will be sequenced through commercial services using standard sequencing primers. Based on the obtained sequence, additional internal primers will be synthesized for further sequencing until the junction of transgene insertion is identified.

To consolidate the identification of two end fragments flanking the transgene insertion, additional λ-phage clones will be isolated by screening the secondary and tertiary pools of clones. Nucleotide sequences obtained from the above clones will be assembled as a single contig. The point of transgene insertion and extent of disruption will be determined. These sequences will be used to search the mouse genome database using a BLAST program and the chromosomal location of the transgene will be identified in *silico* (http://www.ncbi.nlm.nih.gov:80/BLAST). On occasion, transgene insertion causes deletion, duplication or rearrangement of the surrounding genomic regions. Sequence comparisons will be carried out to determine the extent of alteration of the *Jitters* locus in the mutant mouse. If appropriate, BAC clones spanning the region will be obtained from the BAC PAC resource center at Children's hospital Oakland, CA, USA (http://www.chori.org). Potential genes in the vicinity of the transgene insertion will be identified and cDNA clones will be procured (Image consortium). These clones will be useful in probing the genomic organization of the *Jitters* locus.

The purpose of this aim is to establish the nature of the molecular pathway of granule cell degeneration in the *Jitters* cerebellum. We will employ genetic complementation approach to determine the extent of overlap between BDNF pathway and the apoptotic pathway in *Jitters*. Mice heterozygous for BDNF or *Jitters* appear to be relatively normal. If these two pathways intercede, double heterozygotes (BDNF+/-; *Jitters* +/-) should display some of the defects observed in the homozygous Jitters mice (-/-). To test this hypothesis, currently,

we have crossed BDNF+/- and Jitters (+/-) mice and recovered mice with all possible combinations of genotype except BDNF(-/-) which are known to die during early postnatal weeks. Mice that carry Jitters and BDNF mutant allele display some of the behavioral phenotype that includes motor impairment and nervousness. A detailed biochemical and morphological characterization of these mice will be undertaken to ascertain the role of BDNF mediated pathway of cell loss in Jitters.

Mice of different genotypes will be euthanized at different stages and tissues will be collected for analysis. Protein extracts prepared will be subjected to Western blot technique to measure the levels of BDNF, p75 NTR, and GAPDH in the cerebellar tissues. We expect reduced BDNF levels in the compound mutants (BDNF, +/-/*Jitters*, +/-) compared to *Jitters* (+/-) and BDNF(+/-) mice. Morphological evaluation of cerebellum will be carried out to assess the effect of lack of BDNF in *Jitters* (+/-). To address this we will perform TUNEL assays at ages ranging from P1 through 12 weeks of age to look at the pattern of cell death if any in these mice. All possible genotypes will be considered for this analysis. These studies will lead to further discussion of BDNF pathway that affects different aspects of *Jitters* phenotype.

Stakeholder Engagement: None expected.

Scope of Impact:
This project is expected to generate fundamental knowledge critical to animal development and disease. Findings will be published in internationally reputed journals and mouse models generated will be offered to both academic and pharmaceutical researchers to study animal disease processes and for development of therapeutic reagents.

Integrated Activities:
Major findings from these studies will be used as teaching material to under-graduate and graduate courses. Efforts will be made to disseminate knowledge to public through teaching programs in place through Penn State activities.

Milestones:
Significant milestones of these projects will be elucidation of cis-regulators components of Hox genes; establishment of an in vitro system for characterization of trans-acting factors; identification of the nature of Jitters mutant locus; and finally description of the basic molecular pathway disrupted in Jitters. The achievement of these milestones will be monitored annually and the information will be disseminated through scientific publications.

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